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DIAGNOSTICS

# *Mycobacterium tuberculosis* volatiles for diagnosis of tuberculosis by *Cricetomys* rats

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SUMMARY

Tuberculosis (TB) diagnosis in regions with limited resources depends on microscopy with insufficient sensitivity. Rapid diagnostic tests of low cost but high sensitivity and specificity are needed for better point-of-care management of TB. Trained African giant pouched rats (*Cricetomys* sp.) can diagnose pulmonary TB in sputum but the relevant *Mycobacterium tuberculosis* (*Mtb*)-specific volatile compounds remain unknown. We investigated the odour volatiles of *Mtb* detected by rats in reference *Mtb*, non-tuberculous mycobacteria, *Nocardia* sp., *Streptomyces* sp., *Rhodococcus* sp., and other respiratory tract microorganisms spiked into *Mtb*-negative sputum. Thirteen compounds were specific to *Mtb* and 13 were shared with other microorganisms. Rats discriminated a blend of *Mtb*-specific volatiles from individual, and blends of shared, compounds ($P = 0.001$). The rats' sensitivity for typical TB-positive sputa was 99.15% with 92.23% specificity and 93.14% accuracy. These findings underline the potential of trained *Cricetomys* rats for rapid TB diagnosis in resource-limited settings, particularly in Africa where *Cricetomys* rats occur widely and the burden of TB is high.



## 1. Introduction

African giant pouched rats (*Cricetomys* sp.) trained to detect pulmonary tuberculosis (TB) in human sputum have shown potential in TB diagnosis. These rats can accurately detect TB-positive sputum samples[1] by employing their highly developed sense of smell. The training of *Cricetomys* rats for TB diagnosis commences at 4 weeks of age. The training duration required for rats to qualify for diagnosis of TB ranges between 6 and 9 months. Detailed training procedures are described elsewhere.[1] Recent reports[1,2] indicate that such rats can be exploited for TB point-of-care diagnosis in resource-constrained settings. Trained *Cricetomys* rats in use for the past 8 years,[3] show their potential in providing rapid TB diagnosis over prolonged periods of time. Major criteria of satisfactory diagnosis are high sensitivity and specificity, such that the test can diagnose TB patients and rule out healthy individuals. Indeed, a 44% increase in case detection rate was achieved when trained rats were exploited for second-line screening (after smear-microscopy in TB clinics) in Dar es Salaam, Tanzania.[2] The *Mycobacterium tuberculosis* (*Mtb*)-specific volatiles detected by these rats in sputa of TB patients remain largely unknown.[4,5] The purpose of this study was to describe (i) the target volatiles of *Mtb* detected by these rats and (ii) whether other microorganisms related to *Mtb*, which also cause pulmonary disease,[6–9] can be discriminated on the basis of these odour compounds. The volatiles detected by rats were identified using gas chromatography/mass spectrometry.[10] Respiratory tract microorganisms studied included nontuberculous mycobacteria (NTM), different *Nocardia* sp., *Streptomyces* sp., as well as clinical isolates of *Rhodococcus* sp., *Staphylococcus* sp. and *Candida* sp.

## 2. Materials and methods

### 2.1. Culture of microorganisms

Reference strains of *Mtb*, NTM, *Nocardia* sp. and *Streptomyces* sp., as well as clinical isolates (Table 1) were cultured in appropriate

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**Table 1**
Microorganisms used for studying *Mycobacterium tuberculosis* (*Mtb*) target volatiles by GC/MS, including reference strains and clinical isolates from smear-negative *Mtb* culture-negative sputa detected by rats.

| No. | Species | Strain | Source | Medium | Cultures (headspace samples) analysed (n) |
|---|---|---|---|---|---|
| 1 | *Mtb* | H37Rv | Lab strain | b | 3 |
| 2 | *Mtb* | H37Ra | Lab strain | b | 1 |
| 3 | *Mtb* | Beijing 2 | Human, The Netherlands | a, b | 5 |
| 4 | *Mtb* | Beijing 5 | Human, South Africa | a, b, c, d | 23 |
| 5 | *Mtb* | Beijing 6 | Human, Mongolia | a, b, c, d | 9 |
| 6 | *M. smegmatis* | $MC^2155$ | n/a | a, b, c, d | 12 |
| 7 | *M. avium* subspecies avium | n/a | n/a | a, b, c | 4 |
| 8 | *M. scrofulaceum* | n/a | n/a | a, b | 3 |
| 9 | *M. vaccae* | n/a | n/a | a, b | 3 |
| 10 | *M. aichiense* | LMG 19259 | Soil | a, b, c | 8 |
| 11 | *M. aurum* | LMG 19255 | Soil | a, b, c | 9 |
| 12 | *M. neoaurum* | LMG 19258 | Soil | a, b, c | 9 |
| 13 | *Streptomyces antibioticus* | LMG 5966 | Soil | a, b, c | 8 |
| 14 | *S. griseoflavus* | LMG 19344 | Soil | a, b, c | 10 |
| 15 | *S. coelicolor/S. albidoflavus* | DSM 40233 | n/a | a, b, c | 7 |
| 16 | *Nocardia asteroides* | LMG 4062 | n/a | a, b, d | 8 |
| 17 | *Nocardia africana* | DSM 44491 | Human, Sudan | a, b | 6 |
| 18 | *Rhodococcus* sp. | Isolate 61 | Human, Tanzania | e | 2 |
| 19 | *Candida albicans* | Isolate 25 | Human, Tanzania | e | 2 |
| 20 | *Staphylococcus* sp. | Isolate 102 | Human, Tanzania | e | 2 |

a – Middlebrook 7H9 broth; b – Middlebrook 7H11 agar; c – Sauton liquid medium; d – Minimal nutrient PBSGG medium; e – Brain heart infusion (BHI) agar.

culture media including Middlebrook 7H9 broth with ADC enrichment but without Tween and glycerol, Middlebrook 7H11 agar with OADC enrichment only, Sauton liquid medium without glycerol, minimal nutrient medium consisting of phosphate-buffered saline (PBS), glucose and glycerol (PBSGG), and brain heart infusion (BHI) agar. All microorganisms were grown on medium in glass plates or in glass bottles to avoid contamination of the microbial odour by volatiles from plastic materials. Cultures were incubated at 28 °C or 37 °C and controlled for purity especially broth cultures by plating on Luria/Miller (LB) agar incubated at 37 °C before collection of headspace samples for GC/MS analysis.

### 2.2. Analysis of headspace volatile compounds from microorganisms

Headspace samples (volatile compounds in a space above liquid or solid culture) from microorganisms in different media and growth-phase (age) were collected for 18–24 h using a closed-loop stripping apparatus (CLSA) as described by Schulz and co-workers,[11] fitted with an activated charcoal filter (Chromtech; Precision Charcoal Filter, 5 mg). The collected volatiles were eluted from the filter for GC/MS analysis using 30 μl of dichloromethane (Suprasolv, Merck, Germany). GC/MS analysis procedures are described in detail elsewhere.[10]

### 2.3. Specificity of volatile compounds

Volatiles produced by microorganisms grown in different media and during different growth phases were used to establish patterns and clusters of microorganisms and to determine specificity of compounds. The specificity of volatiles was assessed by determining presence and absence in different cultures ($n \geq 2$), in different media, and age (growth in days) of the microorganisms. Volatiles identical to those identified in blank media were excluded. Compounds recovered in at least two cultures were considered significant. Random forest's cluster analysis was performed to construct clusters of microorganisms based on specific and shared volatiles employing pvclust package,[12] using correlation as distance metrics and the average clustering algorithm.

### 2.4. Determination of Mtb target volatiles by Cricetomys rats

The specific volatile compounds responsible for clustering of *Mtb* strains only, *Mtb* and NTM, and *Mtb* and all other microorganisms were selected for determination of TB target volatiles detected by rats in TB sputum samples. The test compounds were assigned to five categories, which enabled testing these compounds alone and in combination: (i) *Mtb*-specific volatiles; (ii) overlapping/shared volatiles from *Mtb*, NTM and others; (iii) combinations (blends) of *Mtb*-specific compounds; (iv) blends of shared volatiles; (v) blends of *Mtb*-specific volatiles plus shared volatiles. The compounds presented to TB detection rats individually or in blends of different compounds (coded 1–31) are shown in Table 2. These compounds were presented in various concentrations ranging from $10^{-1}$ μg/ml to $10^{-5}$ μg/ml. TB-negative sputum samples verified by smear microscopy in TB clinics, which were not detected by trained TB diagnosis rats (also termed as rat-negative) in previous routine TB diagnosis sessions, were spiked with different concentrations of candidate volatiles. The smear-negative sputa not detected by rats has a higher than 93% likelihood of being TB-negative or not containing *Mtb* bacilli.[4] Sputum samples were mixed with 5 ml of PBS and heat-inactivated by boiling at 90 °C, cooled down to room temperature and stored at −20 °C until presented to the rats for TB detection. A minimum of two and a maximum of five different concentrations of candidate volatile compounds were tested by a group of five trained TB detection rats, performing two tests per day. Each sample presented to the rats per test (day) was hence tested 10 times. An interval of 1–2 days was maintained between tests in which rats were subjected to routine TB detection training. The cut-off point of two rats used in routine TB diagnosis by rats was employed whereby the detection of a sample was considered significant when at least two rats detected a given sample. A maximum of 10 correct detections (scores) were expected for each TB-positive control sputum sample as well as potential target volatiles. Nontarget volatiles were not expected to be detected by rats.

Known TB-positive sputum samples with varying numbers of acid-fast bacilli (AFB) counts ranging from AFB 1 to 9, 1+, 2+ and 3+, served as positive controls.[4] Rats were rewarded with food upon correct detection of positive control samples. Food was not

**Table 2**
Volatiles presented to TB detection rats individually and in various combinations (blends).

| Code | Compound name | Source/supplier | Species specificity | Presentation to rats | Cumulative ^ | Rats test* | + Conc. $10^{-1}$–$10^{-5}$ |
|---|---|---|---|---|---|---|---|
| 1 | Methyl nicotinate (99%) | Sigma–Aldrich Chemie | *Mtb*-specific | Individually, different concentration | 16 | – | |
| 2 | Methyl 4-anisate (99+ %) | Sigma–Aldrich Chemie | | | 16 | – | |
| 3 | 2-Phenylanisol (2-Methoxybiphenyl,98%) | Merck | | | 16 | ± | $10^{-2}$ NS |
| 4 | 4-Methylanisol | Merck | | | 16 | – | |
| 5 | Ethyl 4-anisate (97%) | Sigma–Aldrich Chemie | | | 16 | – | |
| 7 | Benzothiazole (96%) | Sigma–Aldrich Chemie | | | 21 | ± | $10^{-2}$ NS |
| 8 | 2-Phenylethanol | Sigma–Aldrich Chemie | Overlapping compounds/found in *Mtb*, NTM and other microbes | Individually, different concentration | 10 | – | |
| 9 | Methyl benzoate (99%) | Sigma–Aldrich Chemie | | | 10 | – | |
| 10 | 4-Pentanolide (γ-valerolactone) (99%) | Sigma–Aldrich Chemie | | | 10 | – | |
| 11 | Methylphenylacetate (99+ %) | Sigma–Aldrich Chemie | | | 10 | – | |
| 12 | Methyl 2-furoate (98%) | Acros Organics | | | 10 | – | |
| 13 | Methyl salicylate (99%) | Acros Organics | | | 10 | – | |
| 14 | Camphor (96%) | Sigma–Aldrich Chemie | | | 10 | ± | $10^{-2}$ NS |
| 15 | Proline + Glycine | Alfa Aesar, Acros Organics, respectively. | *Mtb*-specific | Pairs | 10 | – | |
| 16 | Methyl nicotinate + Methyl 4-anisate | | | | 6 | – | |
| 17 | Methyl nicotinate + 2-Phenylanisol (2-methoxybiphenyl) | | | | 6 | – | |
| 18 | Methyl nicotinate + 4-Methylanisol | | | | 6 | – | |
| 19 | Methyl nicotinate + Ethyl 4-anisate | | | | 6 | – | |
| 21 | Methyl nicotinate + Benzothiazole | | | | 6 | – | |
| **22** | **Methyl nicotinate Methyl 4-anisate 2-Phenylanisol (2-Methoxybiphenyl) 4-Methylanisol Ethyl 4-anisate Benzothiazole** | | **Mtb-specific** | **Combination (blend) of 6 compounds** | **23** | **+*** | **$10^{-3}$ ($P = 0.001$)** |
| 23 | 2-Phenylethanol + Methyl benzoate | | Overlapping compounds/found in *Mtb*, NTM and other microbes | Pairs | 1 | – | |
| 24 | 2-Phenylethanol + Pentanolide (γ-Valerolactone) | | | | 1 | – | |
| 25 | 2-Phenylethanol + Methylphenylacetate | | | | 1 | – | |
| 26 | 2-Phenylethanol + Methyl 2-furoate | | | | 1 | – | |
| 27 | 2-Phenylethanol + Methyl salicylate | | | | 1 | – | |
| 28 | 2-Phenylethanol + Camphor | | | | 1 | – | |
| 29 | 2-Phenylethanol Methyl benzoate 4-Pentanolide (γ-Valerolactone) Methylphenylacetate Methyl 2-furoate Methyl salicylate Camphor | | Overlapping compounds/found in *Mtb*, NTM and other microbes | Combination (blend) of 7 compounds | 6 | – | |
| 30 | Methyl nicotinate Methyl 4-anisate 2-Phenylanisol (2-Methoxybiphenyl) | | *Mtb*-specific | Combination of 3 abundant compounds | 10 | ± | $10^{-3}$ NS |
| 31 | Methyl nicotinate Methyl 4-anisate 2-Phenylanisol (2-Methoxybiphenyl) 4-Methylanisol Ethyl 4-anisate Benzothiazole 2-Phenylethanol Methyl benzoate 4-Pentanolide (γ-Valerolactone) Methylphenylacetate Methyl 2-furoate Methyl salicylate Camphor | | *Mtb*-specific and Overlapping compounds/found in *Mtb*, NTM and other microbes | Combination (blend) of 6 *Mtb*-specific compounds and 7 overlapping compounds | 3 | – | |

+* Detection with statistical significant difference; $P = 0.001$. This cluster is therefore printed in bold.
± Slight detection not statistically significant (NS).
(–) All concentrations were not detected by rats.
+ Conc. = concentrations of volatile compounds detected by rats.
^ The cumulative presentation includes different concentrations of the same compound or combinations.

provided when rats detected any of the negative sputa spiked with test volatiles and the negative control sputum samples, which consisted of confirmed TB-negative sputa that had not been spiked. The overall setup consisted of 70 samples of which 14–18 samples contained various concentrations of test volatiles spiked into negative sputum; 7 TB-positive sputum samples and 42–49 confirmed TB-negative control sputum samples. Fresh smears were made from all detected spiked and negative control sputa to re-assess possible presence of AFB.

### 2.5. Statistical analysis

Clustering of the microorganisms based on volatile compounds was performed using the pvclust package.[12] Significant differences

between the detection of *Mtb*-specific volatiles and shared volatiles by rats as well as differences between detection of different concentrations of volatile compounds were determined using Fisher's exact test at $P$ value $<0.05$.

## 3. Results

### 3.1. Profiles, frequencies and distribution of volatiles

In previous investigations we identified volatiles from various *Mycobacterium* and *Nocardia* species and showed that they are released during culture.[4,10] *Mtb* were more prolific producers of volatiles than NTM analysed. Furthermore, the type of culture medium influenced the compound profiles.[10] In this study, we identified 26 volatiles from microorganisms cultured in different media. Distinct volatiles (see Table 3) were identified from microorganisms in different growth phases (age). Some volatiles were unique to *Mtb* (entries 1–13 in Table 3), whereas others were shared by *Mtb*, NTM and other microbes (entries 14–26 in Table 3).

### 3.2. Clustering of microorganisms according to volatile profiles

Microorganisms were clustered according to their volatiles. Random forest's cluster analysis of these data reveals a significant clustering of *Mtb* strains only; a cluster of *Mtb* and NTM only; and a cluster of *Mtb*, NTM, *Nocardia* sp., *Rhodococcus* sp., *Staphylococcus* sp. and *Candida* sp. (Figure 1). The probability value was highly significant (100%) for these clusters (Figure 1) indicating that clustering was strongly supported by data. The frequencies of occurrence of these volatiles in *Mtb* and other microorganisms are shown in Figure 2, which also depicts the proportion of shared volatiles found in *Mtb* and other microorganisms, including clinical isolates from sputum samples detected by rats. Methyl nicotinate had the highest frequency of occurrence (>51%) in the *Mtb*-specific volatiles. On the other hand, 2-phenylethanol had the highest frequency of occurrence among shared volatiles found in *Mtb* and other microorganisms.

### 3.3. Olfactory detection of Mtb volatiles by Cricetomys rats

A total of seven commercially available volatiles unique to *Mtb* and seven shared volatiles shown in Table 2 were presented to rats in different concentrations, admixed to TB-negative sputum. Compounds were first presented individually to five rats and subsequently in combinations (blends) (Table 2). Rats' detected two *Mtb*-specific volatiles repeatedly (2-phenylanisol and benzothiazole) but their detection was not statistically significant ($P > 0.05$). One shared compound (camphor) was also detected by rats in one out of five tests (days) (50 presentations). Benzothiazole was detected twice by rats with 6 out of 10 scores (60%) on the first day and 7 out of 10 scores (70%) on the second day, but was not detected during the next 5 days, whereas 2-phenylanisol reached 20% or 40% of the expected detection on the fourth and fifth test, respectively. A blend of the three most abundant volatiles (methyl nicotinate, methyl 4-anisate and 2-phenylanisol) was detected in 1 out of 4 days, which was not statistically significant ($P > 0.05$).

The combination of two volatiles, which consisted of methyl nicotinate, and each of the five remaining *Mtb*-specific volatiles (Table 2), as well as a combination of proline and glycine was also not detected by rats in each case. Intriguingly, the combination of

**Table 3**
Frequencies of volatiles unique to *Mtb* or shared with nontuberculous mycobacteria (NTM), *Nocardia* sp., *Rhodococcus* sp., *Streptomyces* sp., *Staphylococcus* sp. and *Candida* sp., from smear-negative/*Mtb* culture-negative sputa.

| No. | Compound* | *Mtb* ($n = 35$) | | | NTM and nonmycobacteria ($n = 56$) | | | |
|---|---|---|---|---|---|---|---|---|
| | *Mtb*-specific volatiles | Present | Absent | Frequency (%) | Present | Absent | Frequency (%) | Tested by rats (TR) |
| Odour compounds unique to *Mtb* | | | | | | | | |
| 1 | Methyl nicotinate | 18 | 17 | 51.42 | 0 | 56 | 0 | TR |
| 2 | Methyl 4-anisate | 17 | 18 | 48.57 | 0 | 56 | 0 | TR |
| 3 | 2-Phenylanisol | 16 | 19 | 45.71 | 0 | 56 | 0 | TR |
| 4 | 4-Methylanisol | 10 | 25 | 28.57 | 0 | 56 | 0 | TR |
| 5 | Ethyl 4-anisate | 9 | 26 | 25.71 | 0 | 56 | 0 | TR |
| 6 | Unknown compound | 8 | 27 | 22.86 | 0 | 56 | 0 | TR |
| 7 | Methyl 2-aminobenzoate | 4 | 31 | 11.42 | 0 | 56 | 0 | n/a |
| 8 | Trimethyloxazole | 3 | 32 | 8.57 | 0 | 56 | 0 | n/a |
| 9 | Benzothiazole | 3 | 32 | 8.57 | 0 | 56 | 0 | TR |
| 10 | 4-Hydroxy-4-methylpentan-2-one | 2 | 33 | 5.71 | 0 | 56 | 0 | n/a |
| 11 | 3-Methyl-4-pentanolide | 2 | 33 | 5.71 | 0 | 56 | 0 | n/a |
| 12 | 4-Methyl-5-hexanolide | 2 | 33 | 5.71 | 0 | 56 | 0 | n/a |
| 13 | Cyclic proline–glycine† | 2 | 1 | 66.66 | 0 | 3 | 0 | TR** |
| Odour compounds shared by *Mtb*, NTM and other respiratory tract microorganisms | | | | | | | | |
| 14 | 2-Phenylethanol | 27 | 8 | 77.14 | 16 | 40 | 28.57 | TR |
| 15 | Methyl benzoate | 23 | 12 | 65.71 | 3 | 53 | 5.35 | TR |
| 16 | 4-Pentanolide | 21 | 14 | 60 | 19 | 37 | 33.92 | TR |
| 17 | Methylphenylacetate | 16 | 19 | 45.71 | 4 | 52 | 7.14 | TR |
| 18 | Methyl 2-furoate | 8 | 27 | 22.85 | 3 | 53 | 5.35 | TR |
| 19 | Dimethyl-pentanolide | 6 | 29 | 17.14 | 2 | 54 | 3.57 | TR |
| 20 | Methyl salicylate | 5 | 30 | 14.28 | 6 | 50 | 10.71 | TR |
| 21 | Camphor | 4 | 31 | 11.42 | 1 | 55 | 1.78 | TR |
| 22 | Methylbutenolide | 2 | 33 | 5.71 | 5 | 51 | 8.92 | n/a |
| 23 | Methyl dimethylbenzoate | 2 | 33 | 5.71 | 2 | 54 | 3.57 | n/a |
| 24 | Benzyl alcohol | 2 | 33 | 5.71 | 6 | 50 | 10.71 | n/a |
| 25 | Ethyl benzoate | 1 | 34 | 2.85 | 2 | 54 | 3.57 | n/a |
| 26 | Aciphyllene§ | | 18 | 0.00 | 7 | 1 | 87.5 | n/a |

* Nawrath et al., (2012).
† Cyclic proline–glycine was obtained in two out of three *Mtb* cultures in PBSGG minimal nutrient medium.
§ Aciphyllene was obtained in 7 out of 8 *Nocardia* cultures in Sauton medium.
** TR tested by rats as combination of proline and glycine.

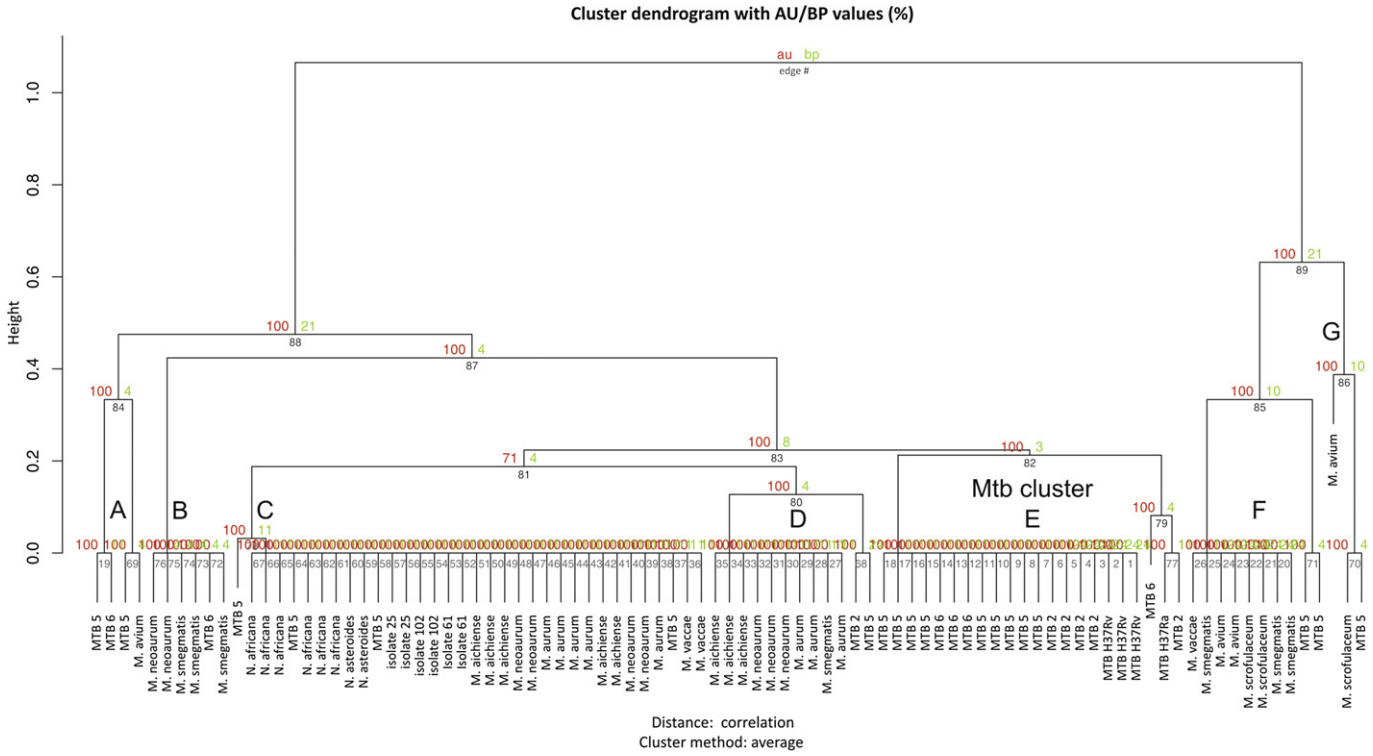


**Figure 1.** Clusters of *Mycobacterium tuberculosis* (*Mtb*), nontuberculous mycobacteria (NTM) and other microorganisms according to odour detection by trained *Cricetomys* rats. Clusters consisting of *Mtb* and NTM (A, B, D, F, and G); cluster of *Mtb*, NTM, *Nocardia* sp., *Candida* sp. (isolate 25), *Staphylococcus* sp. (isolate 102), *Rhodococcus* sp. (isolate 61) (C) and a cluster of *Mtb* strains only (E). *AU* (approximately unbiased) *P-values*, *BP* (bootstrap probability) values.

six *Mtb*-specific volatiles coded 22 (Table 2) was repeatedly detected better than individual compounds (2-phenylanisol and benzothiazole) and the blend of three *Mtb*-specific volatiles (see Figure 3) ($P = 0.001$, Fisher's exact test). The rats detected a medium concentration ($10^{-3}$ μg/ml) of this blend better than higher ($10^{-1}$ and $10^{-2}$ μg/ml) or lower concentrations ($10^{-4}$ and $10^{-5}$ μg/ml) ($P = 0.001$, Fisher's exact test). This medium concentration of the blend of *Mtb*-specific compounds was detected in 4 out of 7 test days. On one occasion, rats detected a slightly higher concentration of the blend of *Mtb*-specific compounds, and the difference to the medium concentration of this blend was statistically significant ($P = 0.001$) (Figure 4).

Of the seven nonspecific volatiles singly tested, only camphor was detected in one out of five tests (not statistically significant). Rats did not detect seven shared compounds, which were also presented in different concentrations as *Mtb*-specific compounds, in 5 test days. The blends of shared volatile compounds (Table 2) were also not detected by rats in repeated tests.

### 3.4. Olfactory detection of TB-positive and TB-negative sputum samples

In sum, rats detected 118 of the 119 positive control sputa included in this study. Rats detected 7 out of 7 TB-positive sputa (100%) presented daily in 16 out of 17 test days. On one occasion, rats detected 6 out of 7 positive control sputa (85.71%) presented. Rats also identified 61 out of 785 negative control sputum samples as TB positive during 17 tests (days) with an average false-positive rate of 7.77% (negative control sputum samples detected by rats). These findings include evaluations performed by all five rats, whereby each test comprised two sessions per rat per sample. Thus, sensitivity of detection of TB-positive sputa was 99.15%, specificity was 92.23% and accuracy was 93.14%.

## 4. Discussion

This study shows that *Mtb* produces specific volatiles that are not produced by NTM and other respiratory microorganisms investigated. Trained *Cricetomys* rats for TB diagnosis detect a blend of the *Mtb*-specific volatiles and hence can be harnessed for the diagnosis of pulmonary TB in sputum samples.

A previous study from our group has shown that *Mtb* produces a variable blend of volatiles depending on growth rate, age, and type of medium.[10] In this study, 13 *Mtb*-specific volatiles were identified repeatedly while 13 compounds were shared with the other microorganisms tested (Table 3). Thus, *Mtb* odour appears distinct from that of other microorganisms, although we cannot formally rule out that these compounds are produced by other microorganisms not included in this study.[13] As indicated earlier,[10] aromatic compounds predominate amongst *Mtb*-specific volatiles (entries 1–9 in Table 3), but lactones (entries 11and 12 in Table 3) are also present.

Trained *Cricetomys* rats can discriminate *Mtb*-specific from shared volatiles. The detection of TB-negative sputa spiked with a blend of six *Mtb*-specific volatiles was significantly different ($P = 0.001$) from TB-negative sputa spiked with shared volatiles, including those from microorganisms isolated from smear-negative, *Mtb* culture-negative sputa detected by rats. This suggests that smear-negative and *Mtb* culture-negative sputa detected by rats probably contain *Mtb* not detected by microscopy, which has lower sensitivity (<60% on average)[14,15] or culture.[16–18] This is supported by recent findings that more than 43% of the culture-negative sputa (25/57 sputum samples) detected by rats had *Mtb* confirmed by Xpert® MTB/RIF (Cepheid Inc.).[19] Individual *Mtb*-specific volatiles of various concentrations were not detected by rats, except 2-phenylanisol and benzothiazole, which were sporadically detected (not statistically significant). Benzothiazole

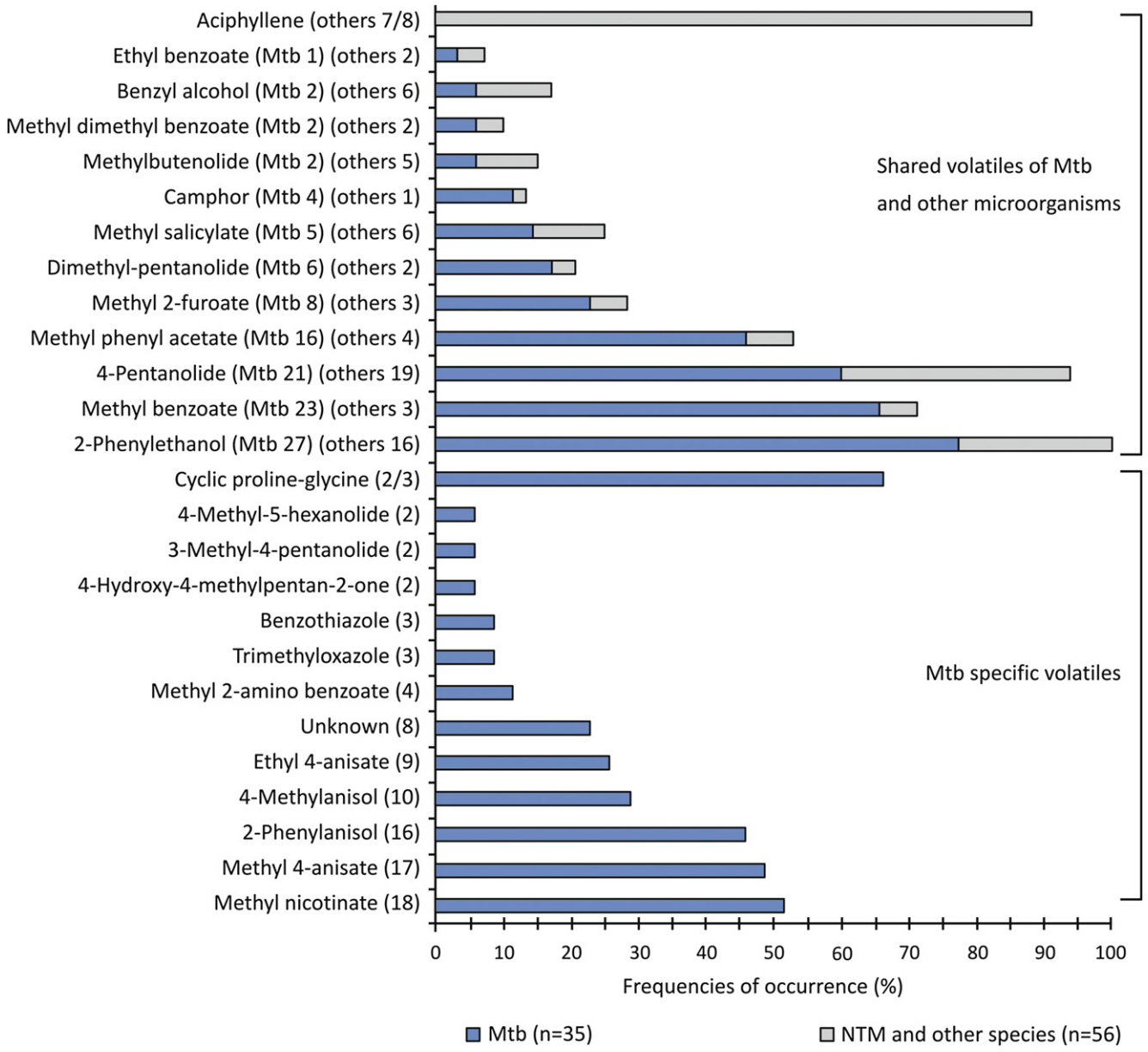


**Figure 2.** Occurrence and proportion of *Mtb*-specific and shared volatiles. Cluster of shared compounds includes *Mtb* strains, NTM, and other microorganisms including *Nocardia* sp., *Rhodococcus* sp., *Streptomyces* sp., *Staphylococcus* sp. and *Candida* sp. Numbers in brackets indicate total sample number including given compound.

was recovered from *Mtb* at early exponential and stationary phases, which were also the growth phases of *Mtb* best detected by rats.[5] Hence, this compound is likely among the major constituents of the detected growth phase of *Mtb* culture. The blend of the three most abundant *Mtb*-specific compounds, methyl nicotinate, methyl 4-anisate and 2-phenylanisol, was sporadically detected but not statistically significant. We conclude that blends of volatiles were better detected by rats than individual compounds. This is in line with the finding that *Mtb* generally produces a greater variety of volatiles than NTM,[10] leading to a chemically unique blend for specific olfactory detection. Indeed, a blend of six *Mtb*-specific volatiles was detected with a statistically higher likelihood than a blend of three abundant *Mtb*-specific volatiles and two individual compounds (Figure 3). We conclude that TB odour detected by rats consists of a unique composition of *Mtb*-specific compounds, rather than a single volatile. Our findings corroborate reports that blends of volatiles elicit positive signals not stimulated by the individual constituents of the blend.[20,21]

Webster and co-workers[22] reported that a given compound (within a blend) presented by itself does not induce olfactory responses in aphid's recognition of a host plant even at the same concentration as a natural sample. Similarly, rats' recognition of natural TB-positive sputa and TB-negative sputa spiked with individual *Mtb*-specific compounds differed. Our findings apparently corroborate results of the aphid study.[22]

The repetitive but inconsistent detection of the blend of *Mtb*-specific compounds (Figures 3 and 4) was probably influenced by not giving a food reward to the rats upon detection of this blend. Reward was not provided to avoid learning of rats to detect test blends and to not disturb routine TB diagnosis by these rats. Variations in the ratios of constituent volatiles may also affect detection, as well as the fact that not all compounds were always

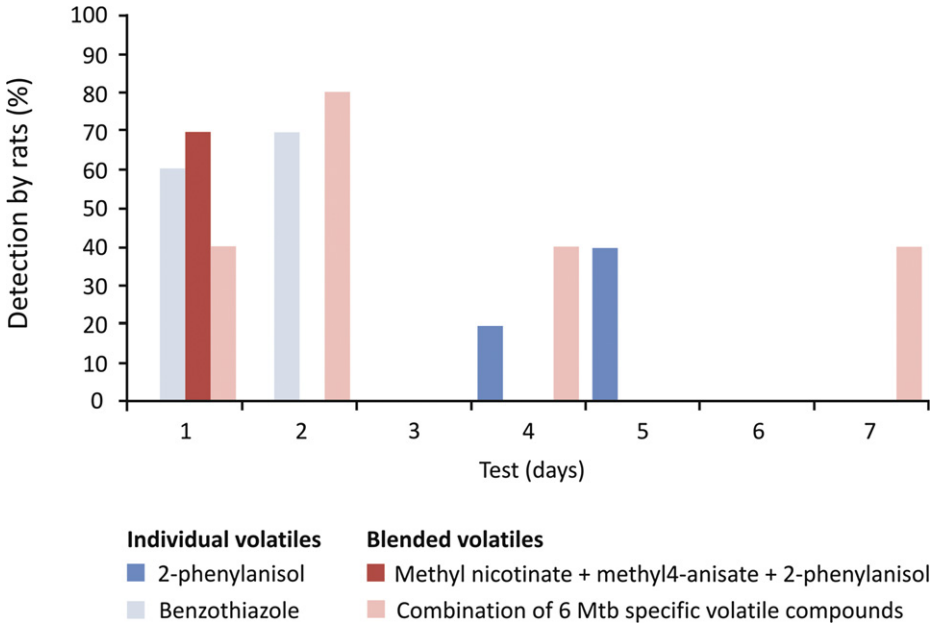


**Figure 3.** Odour recognition of *Mtb*-specific volatiles by rats. The 2-phenylanisol and benzothiazole were slightly detected. None of these compounds were detected when presented in pairs. Combination of the three most abundant *Mtb*-specific volatiles caused slight detection, whereas detection was markedly increased when six *Mtb*-specific compounds were combined (i.e., methyl nicotinate, methyl 4-anisate, 2-phenylanisol, 4-methylanisol, ethyl 4-anisate, methyl 2-aminobenzoate, benzothiazole).

present in all samples. In addition, some *Mtb*-specific compounds were missing in the artificial blend because they were not available to us (Table 3). Nevertheless, our results demonstrate that rats detect even incomplete mixtures of *Mtb* volatiles, more likely to occur under test conditions, and thus respond consistently, even when the composition and ratio of compounds varies in individual samples. The variations found between individual analyses of volatiles, e.g., due to use of different media, are relatively small. This is illustrated by the cluster analysis (Figure 1) where most of the *Mtb* strains fall within a single cluster.

Ratios and constituents of *Mtb* odour produced in host tissue *in vivo* may differ, e.g., due to differences in growth substrates, which determine types of volatiles produced as reported for other microorganisms.[10,23,24] *Mtb* bacilli grown in artificial medium lack the characteristic chemical compounds found in *Mtb* residing in host tissue,[25] and biochemical activities of *Mtb in vitro* or in lung tissue differ markedly.[26] Similarly, the lipid content of *Mtb* grown *in vitro* differs from that of *Mtb* bacilli in lesions.[27] Finally, the gene expression profiles of *Mtb* in lung and in culture medium show profound differences; even expression profiles of *Mtb* derived from different parts of the lung can vary significantly.[28] However, the

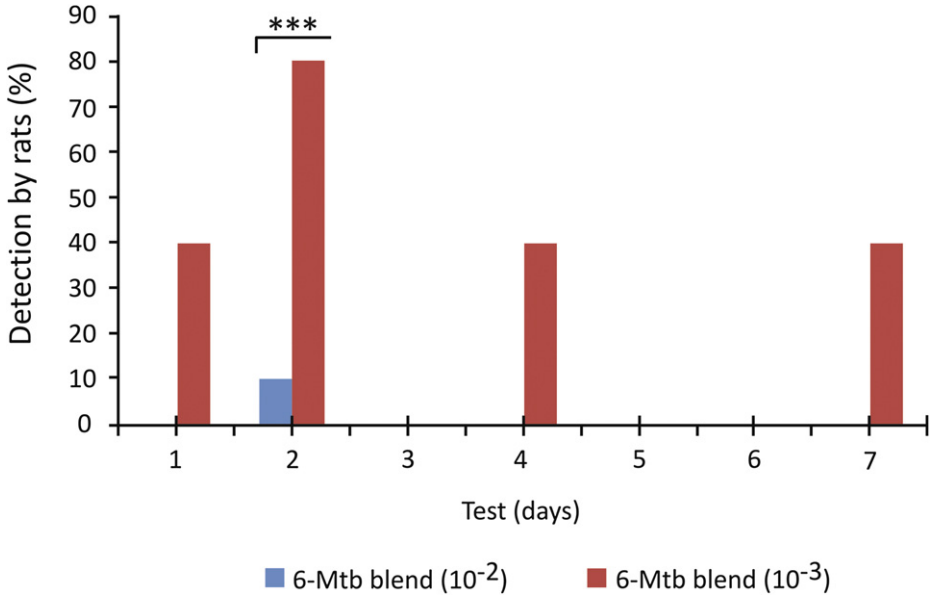


**Figure 4.** Detection of a blend of *Mtb*-specific volatiles by *Cricetomys* rats at medium concentration. Medium concentration ($10^{-3}$ μg/ml; red bars) of blend 22 (Table 3) was repeatedly detected with higher likelihood than higher concentration ($10^{-2}$ μg/ml; blue bar), $P = 0.001$, Fisher's exact test. The medium concentration (red bars) of the *Mtb* blend was detected in four out of seven tests. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

repeated detection of the blend defined here strongly indicates its relatedness to TB odour.

Our study also reveals that the concentration of volatiles is an important factor for detection. A medium concentration of a blend of *Mtb*-specific compounds was significantly better detected than either higher or lower concentrations (Figure 4). Previous reports showed that different concentrations of pheromones evoke distinct behavioural responses, e.g., in mice.[29] Detection of a blend of six synthetic *Mtb*-specific volatiles indicates its relatedness to "natural" TB odour since these rats had not been pre-trained to detect synthetic *Mtb* odour but exclusively natural TB-positive sputa. Our findings further support relatedness of the blend of synthetic *Mtb* odour and characteristic TB odour, considering that TB detection by rats is through learned behaviour that differs from inert behaviour in which the olfactory system responds to specific odour stimuli by default.

Spiking of TB-negative sputa with different volatiles did not affect detection of TB-positive sputa by rats. The sensitivity, specificity and accuracy of odour diagnosis of TB-positive sputa remained higher than 93%.

We conclude that *Mtb* and other microorganisms produce both shared and distinct volatiles. A defined blend of *Mtb*-specific volatiles apparently allows trained rats to discriminate TB-positive from TB-negative sputa for accurate odour diagnosis of TB. Our findings are potentially of general interest for initiatives to develop a point-of-care test for rapid TB diagnosis. Further studies are needed to determine the optimal ratios of the candidate volatiles for improved detection rates and to determine their presence in clinical sputa diagnosed by African giant pouched rats.

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